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published in

The Journal of Neuroscience
2005

DOI (link to publisher)

[10.1523/JNEUROSCI.0977-05.2005](https://doi.org/10.1523/JNEUROSCI.0977-05.2005)

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citation for published version (APA)

Lozovaya, N., Yatsenko, N., Beketov, A., Tsintsadze, T., & Burnashev, N. (2005). Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. *The Journal of Neuroscience*, 25(33), 7499-7506. <https://doi.org/10.1523/JNEUROSCI.0977-05.2005>

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Glycine Receptors in CNS Neurons as a Target for Nonretrograde Action of Cannabinoids

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At many central synapses, endocannabinoids released by postsynaptic cells act retrogradely on presynaptic G-protein-coupled cannabinoid receptors to inhibit neurotransmitter release. Here, we demonstrate that cannabinoids may directly affect the functioning of inhibitory glycine receptor (GlyR) channels. In isolated hippocampal pyramidal and Purkinje cerebellar neurons, endogenous cannabinoids anandamide and 2-arachidonylglycerol, applied at physiological concentrations, inhibited the amplitude and altered the kinetics of rise time, desensitization, and deactivation of the glycine-activated current (I_{Gly}) in a concentration-dependent manner. These effects of cannabinoids were observed in the presence of cannabinoid CB1/CB3, vanilloid receptor 1 antagonists, and the G-protein inhibitor GDP β S, suggesting a direct action of cannabinoids on GlyRs. The effect of cannabinoids on I_{Gly} desensitization was strongly voltage dependent. We also demonstrate that, in the presence of a GABA_A receptor antagonist, GlyRs may contribute to the generation of seizure-like activity induced by short bursts (seven stimuli) of high-frequency stimulation of inputs to hippocampal CA1 region, because this activity was diminished by selective GlyR antagonists (strychnine and ginkgolides B and J). The GlyR-mediated rhythmic activity was also reduced by cannabinoids (anandamide) in the presence of a CB1 receptor antagonist. These results suggest that the direct inhibition of GlyRs by endocannabinoids can modulate the hippocampal network activity.

Key words: glycine receptor; cannabinoids; hippocampus; cerebellum; seizure-like activity; desensitization

Introduction

Cannabinoids influence brain function primarily by activating the G-protein-coupled cannabinoid CB1 receptors (CB1Rs), which are expressed throughout the brain at high levels. Several endogenous lipids, including anandamide (AEA) and 2-arachidonylglycerol (2-AG), have been identified as CB1R ligands (Devane et al., 1992; Di Marzo et al., 1994; Stella et al., 1997). The synthesis of endocannabinoids is triggered by cytoplasmic Ca^{2+} . Endocannabinoids rapidly released from neurons after depolarization mediate some forms of activity-dependent short- and long-term presynaptic modulation of synaptic transmission. These include depolarization-induced suppression of inhibition, depolarization-induced suppression of excitation (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001, 2002; Diana et al., 2002) as well as long-term depression of inhibitory synapses, which selectively “prime” nearby excitatory synapses, facilitating subsequent induction of

long-term potentiation (Chevalere and Castillo, 2004). In particular, in the hippocampus, cannabinoids acting presynaptically modulate both glutamatergic and GABAergic neurotransmission (Misner and Sullivan, 1999; Hajos et al., 2000; Hoffman and Lupica, 2000).

CB1R activation accounts for most of the central effects of cannabinomimetic drugs. Nevertheless, although most of the behavioral effects of cannabinoids are absent in CB1R-deficient mice, cannabinoids (in particular, AEA) still induce catalepsy and analgesia and decrease spontaneous activity in these mice (Di Marzo et al., 1994; Baskfield et al., 2004). Furthermore, in mice, the typical cannabinomimetic effects of AEA on spontaneous activity, body temperature, and pain perception are not reversed by treatment with the selective CB1 receptor antagonist *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide (SR141716A) (Adams et al., 1998). These data indicate the existence of functionally important targets for brain cannabinoid signaling in addition to CB1Rs.

Local alignment of amino acid sequences of cannabinoid receptors and glycine receptor (GlyR) subunits revealed that GlyRs contain few fragments that display a high level of homology with the regions within CB1R and CB2R, which are suggested to be responsible for agonist binding (Mahmoudian, 1997; Tao et al., 1999; Shim et al., 2003) (supplemental Figs. S1–S4, available at www.jneurosci.org as supplemental material). This prompted us to study possible functional interaction between cannabinoid agonists and GlyR.

Received March 12, 2005; revised June 8, 2005; accepted June 12, 2005.

This work was supported by United States Civilian Research and Development Foundation Grant UKB1-2615-KV-04. We thank E. Kondratskaya, A. Fisunov, V. Tsintsadze, C. Holmgren, and A. Brussaard for the helpful comments on this manuscript and O. Krishtal, A. N. M. Schoffeleers, and T. J. de Vries for general support.

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DOI:10.1523/JNEUROSCI.0977-05.2005

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Materials and Methods

Materials. All of the chemicals for intracellular and extracellular solutions were purchased from Sigma (St. Louis, MO). (*R*)-(+)-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo(1,2,3-de)-1,4-benzoxazin-6-yl)-1-naphthalenylmethanone (WIN 55,212-2), AEA, and 2-AG were obtained from Tocris Cookson (Bristol, UK). Ginkgolides B and J were kindly provided by Dr. S. Chatterjee (Dr. Willmar Schwabe Group, Karlsruhe, Germany).

Cell preparation. Wistar rats (12–17 d of age) were decapitated under ether anesthesia, and the hippocampus (or cerebellum) was removed and cut into slices (300–500 μ m) in a solution containing the following (in mM): 150 NaCl, 5 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 1.1 MgCl_2 , and 10 glucose, pH 7.4. Then, the slices were incubated for 10 min (hippocampal) and for 30 min (cerebellar) at 32°C with 0.5 mg/ml of protease (type XXIII) from *Aspergillus oryzae*. Single pyramidal cells from CA1 and CA3 stratum pyramidale layers were isolated by vibrodissociation locally in the stratum pyramidale, and Purkinje cells were isolated by successive trituration of the small pieces of the Purkinje cell layer of the cerebellum through several fire-polished pipettes with opening diameters from 0.5 to 0.1 mm. CA3 and CA1 hippocampal pyramidal and cerebellar Purkinje neurons were identified by their characteristic form and partially preserved dendritic arborization.

After isolation, the cells were usually suitable for recordings for 2–4 h. Throughout the entire procedure, the solutions with the slices were continuously saturated with 95% O_2 and 5% CO_2 gas mixture to maintain pH 7.4. The tested substances were dissolved in DMSO to a stock concentration of 10 mM and kept frozen at -40°C in daily aliquots. The substances were dissolved in external saline to their final concentration immediately before the experiments.

Current recordings. Glycine-activated currents in isolated neurons were induced by the step application of agonists in the “concentration-clamp” mode (Krishtal et al., 1983), using the computerized Pharma-Robot set-up (Pharma-Robot, Kiev, Ukraine). This equipment allows a complete change of saline within 15 ms. Transmembrane currents were recorded using a conventional patch-clamp technique in the whole-cell configuration. Patch-clamp electrodes were pulled with a horizontal puller (Sutter Instruments, Novato, CA) and had an internal tip diameter between 1.4 and 1.8 μ m and a tip resistance between 2.5 and 5 M Ω . The intracellular solution contained the following (in mM): 70 Tris- PO_4 , 5 EGTA, 40 TEA-Cl (tetraethylammonium chloride), 30 Tris-Cl, 5 Mg-ATP, and 0.5 GTP, pH 7.2. The composition of extracellular solution was as follows (in mM): 130 NaCl, 3 CaCl_2 , 5 KCl, 2 MgCl_2 , 10 HEPES-NaOH, and 0.1 μM TTX, pH 7.4. Recording of the currents was performed using patch-clamp amplifiers (Dagan, Minneapolis, MN). To avoid activation of the GABA_A receptor (GABA_AR) by glycine, the GABA_AR antagonist bicuculline (10 μM) was routinely added to all extracellular solutions. Transmembrane currents were filtered at 3 kHz, stored, and analyzed with an IBM-PC computer (IBM Corporation, White Plains, NY) using homemade software. Glycine responses were recorded with a 3 min interval. All experiments were performed at room temperature (19–24°C).

Hippocampal slice experiments. During preincubation, the slices (300–400 μ m thick) obtained from 17- to 19-d-old rats were kept fully submerged in HCO_3^- -buffered artificial CSF (ACSF) as follows (in mM): 135 NaCl, 5 KCl, 26 NaHCO_3 , 1.5 CaCl_2 , 1.5 MgCl_2 , and 20 glucose, pH 7.4, equilibrated with 95% O_2 /5% CO_2 . The experiments were conducted in the same solution, containing 2 mM CaCl_2 and 1 mM MgCl_2 at 32–34°C. The nominally HCO_3^- -free ACSF was saturated with 100% O_2 , and 26 mM NaHCO_3 was replaced with HEPES acid, and pH was titrated to 7.4 with NaOH.

Field potentials were recorded in the stratum radiatum using a tungsten electrode. To stimulate the Schaffer collateral–commissural pathway, a bipolar Ni/Cr electrode was positioned on the surface of the slice. The current intensity of test stimuli (25–50 μA) was set to produce field potentials of half-maximal amplitude. Current pulses were delivered through the isolated stimulator HG 203 (Hi-Med, London, UK) at 0.066–0.2 Hz.

The care and use of animals for all experiments followed the guidelines

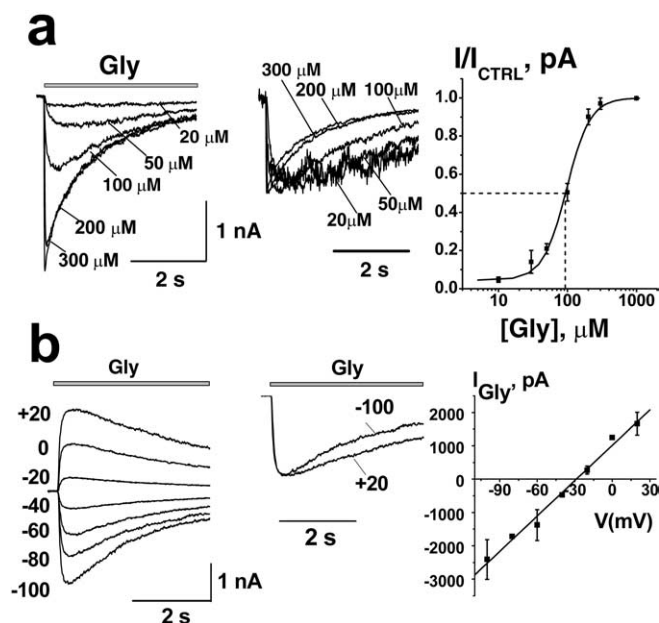


Figure 1. *a*, Agonist concentration dependence of glycine-induced current (I_{Gly}). Left, Family of I_{Gly} representative traces at increasing Gly concentration ranging from 20 to 300 μM . I_{Gly} was recorded in a CA3 hippocampal neuron by conventional whole-cell techniques. Responses were evoked by 4 s application of Gly (indicated here and in subsequent figures by gray bars above traces) at a holding potential of -100 mV in the presence of 10 μM bicuculline. Middle, The same traces normalized to the peak amplitude. Right, Normalized averaged concentration–response curve of I_{Gly} ($n = 4$). *b*, Current–voltage relationship of I_{Gly} . Left, The family of representative traces of current induced by 100 μM Gly at various holding potentials from -100 to $+20$ mV with a 20 mV step. Middle, Traces obtained at -100 and $+20$ mV are superimposed and normalized. Right, I – V of peak I_{Gly} measured with 100 μM Gly.

and protocols approved by our institutional Animal Care and Use Committee (protocol number no2/0204).

Data analysis. The effect of the substance was measured as the mean ratio I/I_{CTRL} , where I is the amplitude of the current under the action of the substance, and I_{CTRL} is the amplitude of the current in control saline. The data were fitted using the Hill equation $I/I_{\text{CTRL}} = 1 - 1/[1 + (\text{IC}_{50}/[\text{S}])^{n_H}]$, where $[\text{S}]$ is the concentration of the substance, IC_{50} is the half-inhibition concentration of the substance, and n_H is the Hill coefficient.

The I_{Gly} desensitization and deactivation kinetics (after 4-s-long pulses) were fitted with monoexponential function. Exponential curve fitting was performed using the simplest algorithm least-squares routines, with single exponential equations of the form $y = y_0 + A_1 e^{-(x - x_0)/\tau}$, where τ_{des} is the time constant of I_{Gly} desensitization, and τ_{deac} is the time constant of I_{Gly} deactivation. The onset kinetics of I_{Gly} was estimated as the 20–80% rise time (t_{on}).

Fourier transformations of 2-s-long epochs of epileptiform discharges were performed using Origin v7.0 software (OriginLab, Northampton, MA).

Data were statistically compared using Student's *t* test at a significance level of $p = 0.05$. Data are expressed as mean \pm SEM.

Results

Glycine-activated currents in isolated hippocampal neurons

Application of glycine to CA1 and CA3 pyramidal neurons isolated from the hippocampus of neonatal rats (12–17 d of age) in the presence of the GABA_AR antagonist bicuculline elicited whole-cell chloride currents in line with previous studies (Krishtal et al., 1988; Shirasaki et al., 1991; Chattipakorn and McMahon, 2002). The amplitude, onset, and desensitization kinetics of glycine-activated currents (I_{Gly}) were dependent on glycine concentration (Fig. 1*a*). The EC_{50} value of glycine was $91 \pm 5 \mu\text{M}$, $n = 4$, with a Hill coefficient of 2.2 ± 0.2 , which corresponds well with the values reported

for acutely isolated cells from the hypothalamus (90 μM) (Akaike and Kaneda, 1989) or acutely isolated hippocampal neurons (69 μM) (Kondratskaya et al., 2002). The reversal potential of I_{Gly} in our experimental conditions was between -35 and -25 mV, with an average value of -28.15 ± 2.2 mV (Fig. 1*b*) ($n = 4$). I_{Gly} was completely and reversibly blocked by specific GlyR blockers strychnine (1 μM) and ginkgolide B (10 μM) (data not shown). These results and the following data were obtained from CA3 pyramidal neurons; however, qualitatively similar results were obtained in CA1 neurons.

Modulation of glycine-activated currents by endogenous cannabinoids in isolated hippocampal neurons

Both endogenous cannabinoids, 2-AG and AEA, at physiological concentrations (0.2–2 μM) strongly inhibited the peak I_{Gly} (Fig. 2*a,b*). $I/I_{\text{CTRL}} (*100\%)$ values were $40 \pm 7\%$ ($p < 0.02$; $n = 4$) with 1 μM 2-AG and $20 \pm 7\%$ ($p < 0.02$; $n = 4$) with 1 μM AEA. Meanwhile, the I_{Gly} onset and desensitization were accelerated in the presence of both endocannabinoids (Fig. 2). For example, the time constant of I_{Gly} desensitization (τ_{des}) decreased to $55 \pm 6\%$ ($p < 0.001$; $n = 4$) of control in the presence of 2-AG and to $40 \pm 15\%$ ($p < 0.02$; $n = 4$) in the presence of AEA. Figure 2 illustrates the averaged ratios of I_{Gly} peak, τ_{des} , and t_{on} values in the presence of 2-AG (Fig. 2*c*) and AEA (Fig. 2*d*) to control. The effects of cannabinoids were concentration dependent (Fig. 2). The peak amplitude, rise time, and τ_{des} of I_{Gly} slowly and partially recovered after washout of both drugs. No changes of the holding current were observed in the presence of cannabinoids.

Endogenous cannabinoids significantly reduced peak and τ_{des} of I_{Gly} at all tested holding potentials. However, the changes in the decay kinetics, induced by 2-AG, were more pronounced at positive membrane potentials (Fig. 3*b,f*). At $+20$ mV, τ_{des} decreased to $31 \pm 9\%$ in the presence of 2-AG compared with $55 \pm 6\%$ at -100 mV ($p < 0.02$; $n = 4$). In contrast, the effects of endocannabinoids on the I_{Gly} onset did not display voltage dependence (Fig. 3*c,g*).

The current decay after removal of Gly represents transitions from active ligand-bound receptor states to inactive unbound states, including channel closure and glycine dissociation. In addition, desensitized receptors may reopen before agonist dissociation, prolonging deactivation (Jones and Westbrook, 1995). The fact that endocannabinoids accelerated the I_{Gly} desensitization suggested the possibility that I_{Gly} desensitization and deactivation are coupled. Indeed, acceleration of I_{Gly} desensitization by 2-AG was accompanied by a significant slowdown of I_{Gly} deactivation (Fig. 3*d,h*). At -100 mV, the deactivation time constant (τ_{deac}) increased to $300 \pm 65\%$ ($p < 0.01$; $n = 3$).

Effects of WIN 55,212-2 on glycine-activated currents

In contrast to endocannabinoids, the synthetic cannabinoid agonist WIN 55,212-2 (1 μM) barely affected the I_{Gly} peak amplitude ($I_{\text{WIN}}/I_{\text{CTRL}}$ was $107 \pm 2\%$; $n = 8$). However, it significantly and reversibly accelerated the desensitization as well as onset of I_{Gly} , in a manner similar to 2-AG and AEA. The desensitization time constant, τ_{des} , decreased to $59 \pm 4\%$ ($p < 0.0001$; $n = 11$) of control in the presence of WIN 55,212-2, and t_{on} decreased to $58 \pm 5\%$ of control ($p < 0.02$; $n = 11$).

The WIN 55,212-2-induced acceleration of desensitization and rise time of I_{Gly} exhibited clear concentration dependence (Fig. 4). It is noteworthy that WIN 55,212-2 at higher concentration induced weak inhibition of the peak currents (at 5 μM , $I_{\text{WIN}}/I_{\text{CTRL}}$ was $65 \pm 5\%$; $p < 0.02$; $n = 5$).

As in the case of endocannabinoids, prominent voltage dependence of the I_{Gly} desensitization observed in control condi-

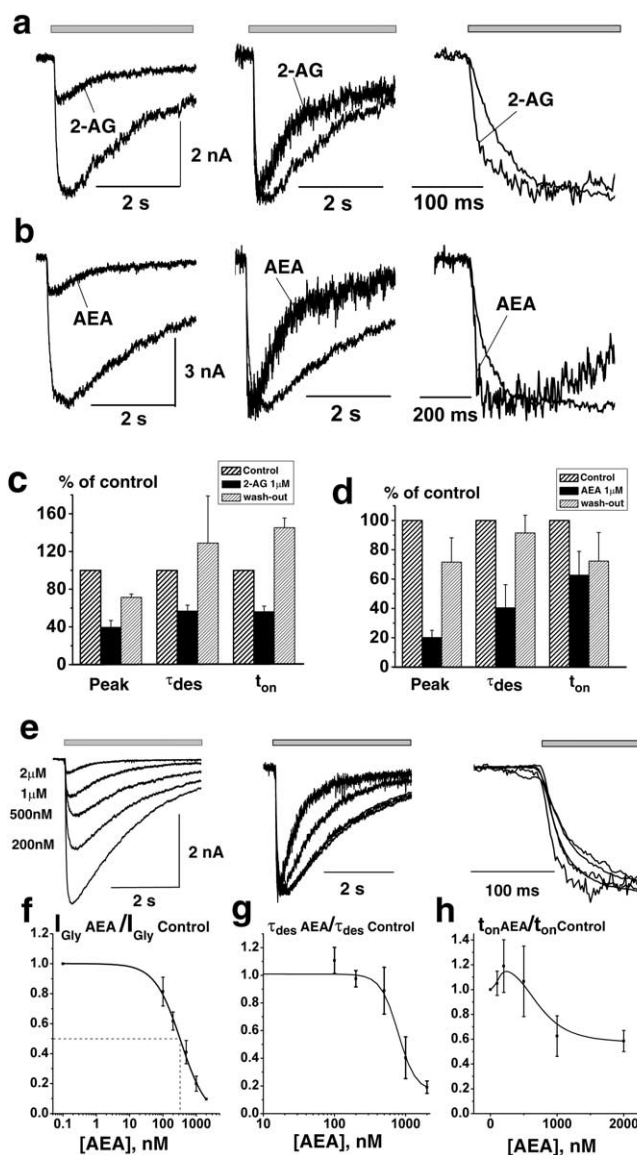


Figure 2. Modulation of I_{Gly} by endogenous cannabinoids. *a, b*, Left, Representative traces of I_{Gly} induced by 100 μM Gly, obtained in control and in the presence of 1 μM 2-AG (*a*) or 1 μM AEA (*b*). Middle and right, Corresponding traces are superimposed and normalized. The averaged numbers for changes of I_{Gly} peak, τ_{des} , and 20–80% rise time (t_{on}) induced by 2-AG (*c*) and AEA (*d*). *e–h*, Concentration dependence of AEA effects on I_{Gly} . *e*, Left, Representative traces obtained at various concentrations of AEA ranged from 200 nM to 2 μM . Middle and right, Corresponding traces are normalized and superimposed. Summary plots showing the effect of increasing AEA concentrations on the amplitude (*f*), τ_{des} (*g*), and t_{on} (*h*) of I_{Gly} are shown ($V_h = -100$ mV; $n = 4$). Data are plotted as a fraction of the maximal glycine current in control.

tions was practically eliminated in the presence of WIN 55,212-2. This corresponds to a much more pronounced effect of WIN 55,212-2 on the desensitization kinetics at depolarized potentials (Fig. 4). Indeed, the decrease of τ_{des} induced by application of WIN 55,212-2 was $35 \pm 5\%$ at $+20$ mV compared with $59 \pm 4\%$ at -100 mV ($p < 0.02$; $n = 4$). However, in contrast to endocannabinoids and despite the pronounced changes in the I_{Gly} desensitization, no significant change of deactivation was observed even with high concentrations of WIN 55,212-2 (data not shown). Qualitatively similar effects of both endogenous and synthetic cannabinoids on GlyRs were observed in isolated Purkinje neurons (supplemental Fig. S5, available at www.jneurosci.org as supplemental material).

The action of cannabinoids on GlyRs is not mediated by activation of CB1/CB3, vanilloid receptors, or G-protein activation

An important question is whether cannabinoid receptors are involved in the modulation of GlyRs. Although CB2Rs are not expressed in the brain (Munro et al., 1993), CB1Rs are abundantly expressed in the CA1 pyramidal layer of the hippocampus (Tsou et al., 1998). In addition to CB1R, a novel “vanilloid-like” CB3 receptor, which shares sensitivity to both cannabinoid and capsaicin receptor agonists, has been demonstrated recently in the hippocampus (Hajos et al., 2001). Furthermore, AEA is known as an agonist of vanilloid receptors, VR1 (Zygmunt et al., 1999, 2000). To test whether cannabinoid and/or vanilloid receptors are involved in modulation of GlyRs, the effects of cannabinoids were tested in the presence of the CB1R antagonist SR141716A or the CB3R antagonist capsazepine. Figure 5 shows that the effects of 1 μ M 2-AG on I_{Gly} peak amplitude, decay, and onset kinetics were preserved in the presence of CB1 receptor antagonist SR141716A (Fig. 5*a,e*). No significant differences in 2-AG or AEA-induced effects on I_{Gly} desensitization and onset were observed in the presence of capsazepine (Fig. 5*c–f*).

To determine whether G-proteins mediate the actions of cannabinoids on GlyRs, the G-protein inhibitor GDP β S was applied to the cells via the pipette solution. After 30 min of GDP β S pretreatment, decay kinetics and rise time were accelerated by 2-AG (1 μ M) to nearly the same extent as in the control [$50 \pm 3\%$ compared with $55 \pm 6\%$ in control ($n = 3$; $p > 0.2$) for τ_{des} ; $68.5 \pm 3\%$ compared with $56 \pm 5\%$ in control (Fig. 5*b,e*) ($n = 3$; $p > 0.4$) for t_{on}]. Inhibition of I_{Gly} peak amplitude was also not significantly different from control numbers ($52 \pm 2\%$ compared with $40 \pm 7\%$ in control; $n = 3$; $p > 0.2$). Altogether, these data are consistent with a direct action of cannabinoids on GlyRs. In addition, the mapping studies in rat brain showed that CB1Rs are mainly localized to axons and nerve terminals and are essentially absent on the neuronal soma or dendrites (Tsou et al., 1998).

GlyR-driven seizure-like rhythmic activity in hippocampal slices

The function of GlyRs in the hippocampus is not clear. It is possible that these receptors mediate neuronal function in a manner similar to GABA $_A$ Rs. Numerous reports indicate that GABA $_A$ Rs and GlyRs, principal inhibitory receptors, when in-

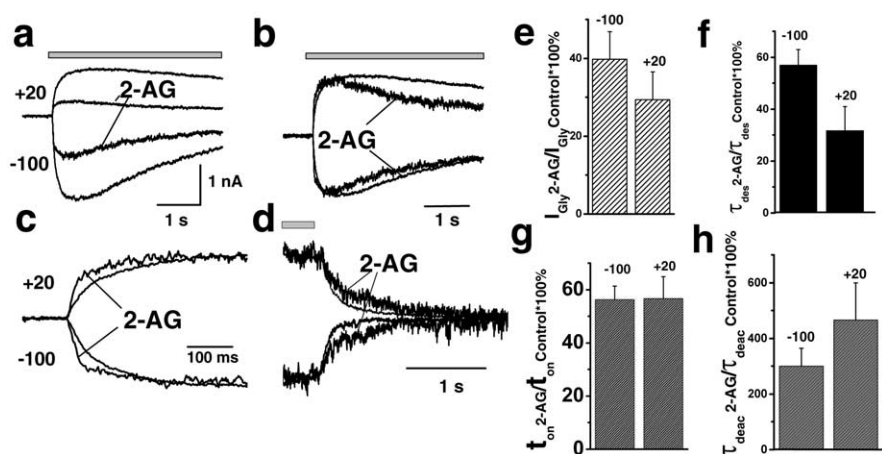


Figure 3. Voltage dependence of the I_{Gly} modulation by 2-AG. *a*, Representative traces obtained in control condition and in the presence of 1 μ M 2-AG at holding potentials of -100 and +20 mV. *b*, *c*, Corresponding traces normalized and superimposed. *d*, Deactivation of I_{Gly} before and after preapplication with 2-AG. *e–h*, Statistics for the 2-AG effects on peak amplitude (*e*), τ_{des} (*f*), t_{on} (*g*), and τ_{deac} (*h*) obtained at holding potentials of -100 and +20 mV.

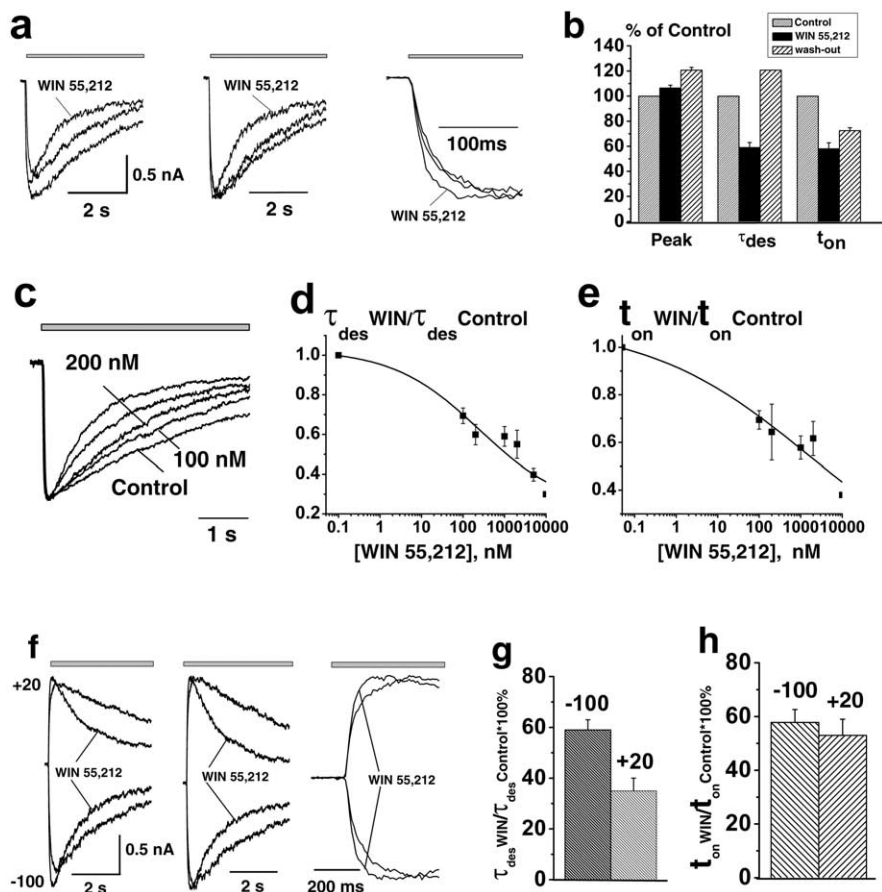


Figure 4. Modulation of glycine-activated currents by WIN 55,212-2. *a*, Left, Traces of I_{Gly} induced by 100 μ M Gly obtained in control, in the presence of WIN 55,212-2 (1 μ M), and after washout of drug (gray line) ($V_h = -100$ mV). Middle and right, The same traces are superimposed and normalized. *b*, Statistics of the effects of WIN 55,212-2 on the amplitude, τ_{des} , and t_{on} ($n = 11$) of I_{Gly} . *c–e*, Concentration dependence of the WIN 55,212-2 effects on I_{Gly} kinetics. *c*, I_{Gly} traces recorded at various concentration of WIN 55,212-2 ranged from 100 nM to 2 μ M measured in the same cell are normalized and superimposed. *d*, *e*, Summary plots showing the effect of increasing the WIN 55,212-2 concentrations on τ_{des} (*d*) and t_{on} (*e*) measured at $V_h = -100$ mV ($n = 4$). Data are plotted as a fraction of the maximal glycine current in control. *f–h*, Voltage dependence of the I_{Gly} modulation by the WIN 55,212-2. *f*, Left, I_{Gly} representative traces obtained in control condition and in the presence of 1 μ M WIN 55,212-2 at holding potentials of -100 mV and +20 mV. Middle and right, The same traces are superimposed and normalized. Statistics for the WIN 55,212-2 effects on τ_{des} (*g*) and t_{on} (*h*) at holding voltages of -100 and +20 mV.

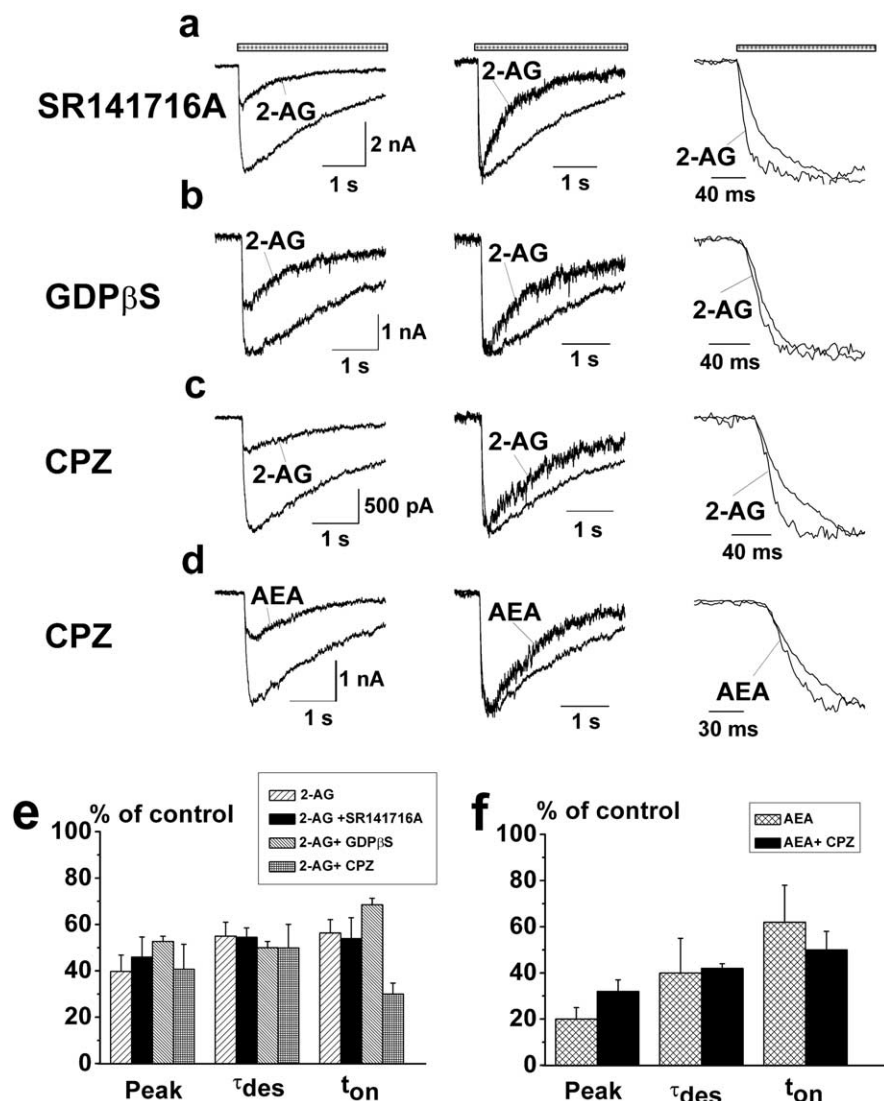


Figure 5. CB1/CB3 receptors and G-proteins are not involved in the modulation of I_{Gly} by cannabinoids. **a–c**, Left, Traces of I_{Gly} (100 μ M) obtained in the control and with 1 μ M 2-AG in the presence of SR141716A (2 μ M) (**a**), intracellularly preapplied GDP β S (2 mM) (**b**), and capsazepine (CPZ; 1 μ M) (**c**). $V_h = -100$ mV. Middle and right, The same traces are superimposed and normalized. **e**, Statistics of the effects of 2-AG on the peak amplitude, τ_{des} , and t_{on} ($n = 4$) of I_{Gly} in the presence of SR141716A, capsazepine, and during intracellular perfusion with GDP β S. I_{Gly} peak amplitude inhibition induced by 2-AG in the presence of SR141716A was $46 \pm 9\%$ compared with $40 \pm 7\%$ in control ($n = 3$; $p > 0.5$), whereas corresponding figures for τ_{des} were $55 \pm 4\%$ in the presence of antagonist compared with $55 \pm 6\%$ ($n = 3$; $p > 0.8$), and t_{on} was $54 \pm 9\%$ compared with $56 \pm 5\%$ ($n = 3$; $p > 1$). **d**, Left, Traces of I_{Gly} (100 μ M) obtained in the control and with 1 μ M AEA in the presence of 1 μ M capsazepine (CPZ). Middle and right, The same traces are superimposed and normalized. **f**, Statistics of the effects of AEA on the peak amplitude, τ_{des} , and t_{on} of I_{Gly} in the presence of CPZ. In the presence of CPZ, decay kinetics and rise time were accelerated by AEA to nearly the same extent as in the control: $42 \pm 2\%$ compared with $40 \pm 15\%$ in control ($n = 3$; $p > 0.8$) for τ_{des} ; $50 \pm 8\%$ compared with $62 \pm 16\%$ in control ($n = 3$; $p > 0.7$) for t_{on} .

tensely activated, excite rather than inhibit neurons (Alger and Nicoll, 1979; Staley et al., 1995; Kaila et al., 1997; Taira et al., 1997; Backus et al., 1998). It has been shown that excitatory GABAergic transmission seems to play an active functional role in the generation of seizure-like rhythmic activity in the hippocampus (Grover et al., 1993; Staley et al., 1995). We hypothesized that in conditions of intense activation, GlyRs could also participate in processes of synchronization of neuronal activity. To address the functional implication of the direct action of cannabinoids on GlyRs in the hippocampus, we tested the possibility that GlyRs are involved in seizure-like activity induced by high-frequency

stimulation in the presence of bicuculline and, if so, whether they are then modulated by application of cannabinoids.

CA1 field network activity was induced by repetitive stimulation with short bursts of high-frequency stimulation (seven stimuli at 50 Hz) applied to the Schaffer collateral–commissural pathway with a 4 min interval in the presence of bicuculline (20–40 μ M) (Fig. 6). This type of stimulation protocol imitates natural patterns of activity (Ranck, 1973). Seizure-like events lasted for 1–4 s after burst stimulation and can be classified as intermediate ictal activity (>500 ms, interictal; <4 s, ictal) (Traub et al., 1996). Experiments in which the CA3 zone was surgically cut revealed that this type of activity originated in the CA3 zone, being completely eliminated in minislices (Fig. 6a). Seizure-like activity was completely blocked by antagonists of excitatory transmission, 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline-7-sulfonamide (20 μ M) or D-AP-5 (50 μ M; $n = 5$). Our experiments revealed that seizure-like activity was disrupted by strychnine (5–10 μ M) (Fig. 6b) and selective GlyR antagonists ginkgolide B (10–20 μ M) and ginkgolide J (50–70 μ M) (data not shown). Correspondingly, the power of hippocampal epileptiform activity decreased in the presence of antagonists (Fig. 6e,h).

It has been shown that the GABA $_A$ -mediated excitatory response is a result of an asymmetric, activity-dependent collapse of the opposing electrochemical gradients of two anions (Cl^- and HCO_3^-) that permeate a GABA $_A$ channel (Bormann et al., 1987; Kaila and Voipio, 1987). We presumed that depolarizing, GlyR-activated, HCO_3^- -carried currents, as in the case of GABA $_A$ Rs (Bormann et al., 1987; Staley et al., 1995; Kaila et al., 1997), could be involved in the generation of strychnine-sensitive seizure-like activity. To prove this hypothesis, we conducted experiments in nominally CO_2/HCO_3^- -free medium. After 20–30 min of perfusion with HEPES-buffered solution saturated with 100% O_2 , seizure-like activity was strongly attenuated (Fig. 6c,f,i). This observation is

consistent with a key role for HCO_3^- in the generation of the depolarizing response.

Modulation of hippocampal GlyR-driven epileptiform activity by cannabinoids

In line with strong inhibitory action of cannabinoids on I_{Gly} observed in isolated neurons, GlyR-driven epileptiform activity was also significantly impaired by high concentrations of AEA (10–20 μ M) in control conditions and in the presence of CB1 receptor antagonist SR141716A (Fig. 6d). The maximal power of hippocampal epileptiform activity (frequency, ~ 10 Hz) decreased in

the presence of AEA in control to $31 \pm 9\%$ ($n = 4$) compared with $54 \pm 8\%$ ($n = 4$), in the presence of SR141716A (Fig. 6*g,j*). Concentrations of AEA used in this set of experiments were higher than those in experiments using isolated cells to overcome the enzymatic attack of fatty acid amide hydrolase, which breaks down anandamide into arachidonic acid and ethanolamine (Hillard et al., 1995).

WIN 55,212-2 at a high concentration ($10 \mu\text{M}$), which produced significant inhibition of I_{Gly} amplitude, attenuated seizure-like activity in the hippocampus in the same way as AEA (data not shown). These results suggest the possibility of modulation of the GlyR-mediated network activity by cannabinoids.

Discussion

Here, we report that cannabinoids strongly modulate gating of GlyR-activated chloride channels in isolated hippocampal pyramidal and Purkinje neurons. Thus, in addition to the well known retrograde mode of cannabinoid action activating presynaptic CB1Rs (which implies diffusion of a messenger from postsynaptic elements backwards to the presynaptic cell), the present results show a novel, direct action of endocannabinoids on GlyRs. These receptors can be located in close proximity to the point of release of cannabinoids from the postsynaptic cell and may serve as a target for cannabinoid signaling.

Glycinergic synapses are found in many CNS regions, including the spinal cord, brainstem, and cerebellum (Jonas et al., 1998; Chery and de Koninck, 1999; O'Brien and Berger, 1999), where they play a well established role in the processing of motor and sensory information that controls movement, vision, and audition (Aprison, 1990; Moss and Smart, 2001). Our results suggest that this transmission can be directly modulated by endogenously released cannabinoids. The machineries necessary for both synaptic and nonsynaptic GlyR-mediated transmission have been reported to be present in the hippocampus. In particular, synaptoneuroosomes obtained from adult rat hippocampus contain glycine and release it by both vesicular and nonvesicular mechanisms (Burger et al., 1991; Engblom et al., 1996). Expression of β heteromeric GlyRs in the developing hippocampus (Malosio et al., 1991; Chattipakorn and McMahon, 2002; Kondratskaya et al., 2004) indicate the synaptic location of GlyRs, because the β subunit is required for receptor clustering (Kirsch et al., 1993; Meyer et al., 1995). Recent immunocytochemical studies demonstrate both synaptic and nonsynaptic expression of GlyRs in the hippocampus (Brackmann et al., 2004). Finally, colocalization of glycine transporters and GlyRs in

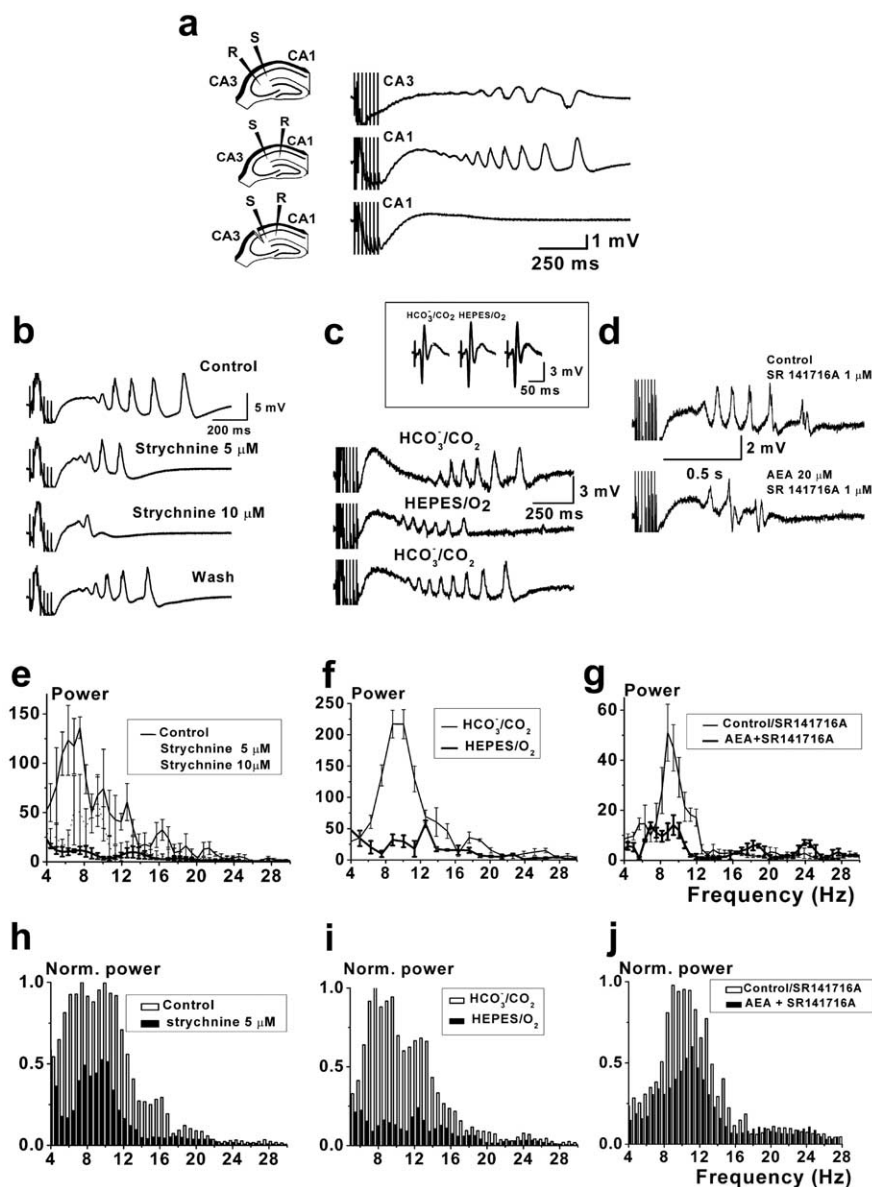


Figure 6. GlyR-driven seizure-like rhythmic activities in hippocampal slices attenuated by AEA. *a*, Seizure-like activity recorded from CA1 and CA3 stratum radiatum in response to stimulation (S) of Schaffer collateral–commissural pathway with high-frequency short trains of stimuli (7 stimuli; 50 Hz) applied every 4 min in the presence of $30 \mu\text{M}$ bicuculline. Slice events were detected by using extracellular field potential recordings (R). Cutting the Schaffer collaterals abolishes discharges in the CA1 area, indicating that excitatory inputs from the CA3 area are necessary for entraining CA1 to generate the epileptiform discharges. Traces of epileptiform discharges in control and in the presence of strychnine (*b*) and AEA ($20 \mu\text{M}$) against the background of SR141716A ($1 \mu\text{M}$) (*d*) are shown. *c*, Experiment demonstrating attenuation of epileptiform activity after 30 min of perfusion in nominally CO_2 -free superfusion medium. Note that control field potentials, evoked by single stimuli stimulation of Schaffer collateral–commissural pathway (no bicuculline), did not change after changing of superfusion medium (inset). *e–g*, Corresponding averaged Fourier transformations (4 epochs of 2 s recordings) of epileptiform activity. *h–j*, The normalized summarized Fourier transformations of 2-s-long epochs of epileptiform discharges at β -frequency bands in control and in the presence of strychnine ($5 \mu\text{M}$; $n = 5$) (*h*), in CO_2 -free medium ($n = 3$) (*i*), and in the presence of AEA and SR141716A ($n = 4$) (*j*).

the hippocampus (Jursky and Nelson, 1995) suggests the possibility that extrasynaptic GlyRs could be activated by reverse uptake of glycine. Few reports suggest the possibility that GlyRs may mediate the inhibitory effects of glycine in the hippocampus (Cherubini et al., 1981; Seiler and Sarhan, 1984; Chattipakorn and McMahon, 2003). Nevertheless, to date, the physiological role for and conditions under which GlyRs are activated in the hippocampus were unknown.

Our data show that seizure-like activity induced by repetitive

high-frequency short-burst stimulation in the presence of bicuculline in the CA1 zone of hippocampal slices revealed paradoxal sensitivity to GlyR antagonists, indicating that GlyRs drive this activity. We suggest that GlyRergic excitation can participate in the expression of seizure-like rhythmic synchronization in the hippocampus. This hypothesis was supported by our finding that strychnine-sensitive slice events were eliminated in CO_2/HCO_3 nominally free medium, indicating that HCO_3 ions are the major driven anions for seizure expression. Therefore, it is possible to suggest that inhibition of GlyRs induced by cannabinoids could result either in a decrease in the amount of synaptic GlyR-mediated excitatory drive necessary for synchronized behavior or, if extrasynaptic GlyRs are the main players, in the decrease of tonic excitability and shift of seizure threshold. Thus, in either the extrasynaptic or synaptic scenario, inhibition of GlyRs would lead to a disruption of network activity. Indeed, GlyR antagonist-sensitive epileptiform activity was depressed by AEA. The latter finding is in agreement with the previous observation that both WIN 55,212-2 and AEA inhibited interictal activity induced by low- Mg^{2+} /high- K^+ medium recorded in the CA1 stratum pyramidale of the hippocampus (Ameri et al., 1999).

Acceleration of desensitization of I_{Gly} by cannabinoids is of particular interest. Both homo-oligomeric and hetero-oligomeric GlyRs display slow desensitization with a slow recovery. This suggests that the time course and the amplitude of glycinergic miniature IPSCs will only be slightly affected by desensitization. Desensitization of homomeric $\alpha 1$ GlyR has been shown by others to be more prominent with increased receptor density (Legendre et al., 2002) and with phosphorylation of the receptor (Gentet and Clements, 2002). In both of these studies, acceleration of desensitization was reported to prolong the current relaxation time course. Slow desensitization of GlyRs cannot play a significant role after release of a single vesicle. However, it might contribute to glycinergic synaptic efficacy when a long-lasting (seconds-range) depolarization of the postsynaptic membrane is coupled with high-frequency cell activity (Legendre, 1998). Moreover, the prolonged deactivation phase of glycinergic events should act to reinforce the efficacy of inhibition.

Slowdown of I_{Gly} deactivation could also have important consequences for inhibitory network behavior, because the rate of decay of synaptic responses can significantly alter firing frequency and the ability of the network to synchronize (Wang and Rinzel, 1992; Wang and Buzsaki, 1996).

The time course of synaptic conductance is an important determinant of the temporal precision of information processing within a neuronal network. Synaptic integration depends on the duration of the EPSPs, because this determines both the time window within which they summate to reach spike threshold and the temporal precision of spike generation (Fricker and Miles, 2000; Galarreta and Hestrin, 2001) and the efficacy of information transfer (London et al., 2002). This is important because fast EPSPs allow neurons to behave as coincidence detectors, whereas neurons with slow EPSPs may behave as temporal integrators (Geiger et al., 1997; Trussell, 1997; Taschenberger and von Gersdorff, 2000). In particular, interaction between excitatory and inhibitory action provides a mechanism for processing auditory temporal information. Blocking GABAergic or glycinergic inhibition can eliminate duration tuning, suggesting that the neural code for sound duration is the result of convergence of excitatory and inhibitory inputs (Casseday et al., 2000). The matching of excitatory transmission in the calyx of Held by a powerful, precision glycinergic inhibitory system suggests that the relay function

of the medial nucleus of the trapezoid body of rats may be rapidly modified during sound localization (Awatramani et al., 2004).

In the case of the “extrasynaptic scenario,” prolonged depolarization induced by tonic activation of extrasynaptic GlyRs would be decreased in the presence of cannabinoids, resulting in decreased neuronal excitability. Inhibition of tonic GlyR-mediated conductance can lead to attenuation of membrane conductance and slowdown of the EPSC decay that is crucial for temporal summation.

Altogether, our findings suggest a fundamental importance of GlyR in hippocampal network activity and nominate GlyR as a novel target for endocannabinoid signaling.

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